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Cellular immune reactions in the lung

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Summary:

The lung constantly interacts with the environment through thousands of liters of air that are inhaled daily. This continually transports toxic chemicals and particles or pathogenic microorganisms deep into the respiratory system posing a challenge to physicochemical barriers and the local immune system. Thus complex structures and mechanisms have evolved to recognize and fend off environmental dangers while at the same time allowing efficient gas exchange. Here we review our current knowledge regarding cellular mechanisms of the immune system in context with the highly specialized anatomical features of the airways and especially the alveolar compartment. The focus will be on fungal and viral infections, attempting to merge anatomical aspects well known to pulmonologists with fundamental immunological concepts. We discuss the specialized morphological constraints of immune cells compressed under a continuous layer of the surfactant lining within alveoli as well as the importance of functional polarization of respiratory tract epithelia. Furthermore we summarize the different types of innate and adaptive immune cells and their relative contribution to lung homeostasis with respect to localization. Finally we provide a list of currently unresolved questions with high relevance for the field that might serve as food for thought regarding future research directions.

Structure and immunological threat of the airways

The respiratory apparatus of mice and humans consists of nose, oropharynx, larynx, conducting airways and the actual respiratory surface. Despite only containing a volume of ~ 5 liters in man, the total respiratory surface of the lung exceeds 120 m² (1), so more than 30 times the body surface. This is due to the presence in lung of millions of small alveoli, spheroid sacs at the terminal end of the conducting airways that provide an extremely thin epithelium which is optimized for the diffusion of respiratory gases. A recent re-estimation of the total number of alveoli in the human lung has found a mean of 480 million units with a remarkably narrow size distribution around $4.2 \cdot 10^6 \mu\text{m}^3$, which equals an alveolar radius of ~100 μm (2). Thereby alveoli do not assume the shape of a perfect sphere. Instead they present as polyhedron (3,4) with sharp edges, since this geometrical form can assume a much better ratio of internal surface to a given volume, a critical issue for an organ having to provide as much respiratory surface per volume, as possible. Indeed, the inner surface of the lung is twice as big as it would be when the 480 million alveoli would be perfect spheres.

During rest a typical human inhales 500 ml of air per breath at a rate of 13-17 inhalations per minute. This accounts for 10-12,000 liters of air per day (5). However, during exercise or increased physical labor this rate can easily increase tenfold (6). Despite containing molecular oxygen as the central component taken up for respiration, normal breathing air consists of many other gaseous components but also carries a plethora of particulate matter, the amount of which is strongly dependent on the environment. Thus, while the air in the Antarctic will approach clean room quality with hardly more than just atmospheric gases, air in traffic loaded inner city main streets contains huge numbers of exhaust particles from engines or other sources of combustion as well as chemicals and aggressive materials such as asbestos (7-10). Apart from these man made sources of pollution air can carry large amounts of fungal spores, bacteria, viruses or allergens such as pollen and house dust (11), which can cause localized infectious outbreaks, especially when transported over inadequate artificial ventilation systems as e.g. in commercial airliners (12,13) or overcrowded jails (14). Despite being already very high, such numbers are still toppled by the amount and chemical aggressiveness of substances that are contained in cigarette smoke (15,16).

Thus, there is a continuous intense confrontation of the large surface of the respiratory tract with airborne noxious threats and potentially pathogenic microorganisms. As a result the mucosal tissue in the nasal passages and oropharynx is always colonized by a multitude of bacteria. It remains, therefore, an amazing achievement that under normal conditions behind the larynx the airways and the respiratory surface of the alveoli are sterile (17). And although

smoking will eventually lead to the breakdown of lung physiology accompanied by life threatening conditions, this may well take years to decades of intense smoking to occur (17).

To constantly monitor and sustain sterility of the lung an effective system of surveillance and cleaning has evolved which is brought about by a unique design of the conducting airways and the alveoli. Starting in the nose a coarse filter consisting of hair and mucus will interfere with the entry of material exceeding a certain size limit. Combined with a rapid sneezing reflex potentially hazardous or allergic material will immediately be removed from the airways or trapped in the mucus. Despite being very sticky and viscous the mucus also contains many antibiotic factors such as antimicrobial peptides or oxidizing enzymes (18). Thus, mucus not only constitutes a physical trap, it also has considerable antibiotic activity. The airways themselves are formed by specialized epithelial cells that contain a ciliated surface. By their rhythmic beat the cilia can transport mucus-ensheathed particles from deeper parts of the conducting airways to the outside. This transport mechanism (“mucociliary escalator” (17)) is extremely important for the maintenance of lung sterility. Cigarette smoke (15,19) or congenital defects such as primary ciliary dyskinesia (20) can interfere with or completely disrupt the escalator mechanism which can result in inflammation and recurrent infections. In addition to the ciliary escalator airway epithelial cells also form a continuous layer between interstitial tissue and the airspace by sealing cell borders with tight junctions (17). The breakage of this barrier, e.g. by influenza-mediated epithelial cell death, is associated with increased pathology of bacterial superinfections (21).

While these biophysical mechanisms are obviously well suited to maintain the safety and cleanliness of the conducting airways, neither thick mucus nor ciliated epithelia can be found on the inner face of alveoli, although these have a total surface that exceeds that of all other airways combined by three orders of magnitude. This is for the obvious reason that alveoli need to provide an extremely thin plane that is optimized for the diffusion of the respiratory gases oxygen and CO₂. Since the free diffusion distance of oxygen through tissues is only fractions of a millimeter the distance between the membrane of an erythrocyte in a pulmonary capillary and the air space in an alveolus must not exceed 1 μm (3) on average to allow effective oxygenation of flowing blood.

The overall structure of the lung is extremely delicate and optimized for providing a large interaction surface of atmospheric gas with the blood to effectively draw in oxygen and discard CO₂ from the body. From the main trachea conducting airways branch off in thinner conducting airways called bronchi. After 14 branching generations terminal bronchioles are reached that lead via a transitional into respiratory bronchioles, then alveolar ducts which

each finally end in a number of alveoli (Fig. 1A/B) as 23rd generation on average (1). Beginning with transitional bronchioles also airway ducts have multiple alveoli attached to their sides, which is why respiration starts at this level of airways (1). The sum of all lower bronchioles fed by one 15. generation transitional bronchiole constitutes a pulmonary acinus (Fig. 1B) and contains approx. 10.000 alveoli (3).

The design of an alveolus directly reflects its main function as a respiratory surface. It is covered by two types of alveolar epithelial cells (AECs), type I and type II. While type I cells only make up 1/3 of all cells in the alveolus in number, they cover 97% of its surface due to the fact that each cell is spread out over 5,000 μm^2 . Thus, type I AECs provide the thin respiratory surface of an alveolus. Type II AECs in contrast are almost round in appearance and contain so called lamellar bodies (Fig. 1C). They are storage sites for surfactant, a thin liquid film that is constantly produced by type II AECs (22), covers the entire surface of the alveolus and has important functions for the biology of the lung (see below). AECs II are considered precursors for type I AECs and can replace them at sites of alveolar damage (3). Individual alveoli are separated from each other by thin septae, within which the capillaries of the pulmonary blood vessels are flowing. By this design it is ensured that each blood vessel has contact to the ventilated surface of two alveoli, one from each side (1,3). Importantly, alveoli are connected to each other by multiple holes within the septae, so called pores of Kohn (Fig. 1C). The surfactant layer of alveoli is continuing through these pores and material in the liquid hypophase below the surfactant layer but not air can freely distribute through them (23). Our own observations also suggest that immune cells recruited to the surface of alveoli can migrate through these pores (24), which had been hypothesized already from electron microscopic studies (23).

Immunological control of alveolar integrity - Surfactant

The mucociliary escalator and other mechanisms discussed above very effectively clean the incoming breathing air. Nevertheless, it is still possible for pathogens, e.g. pneumococci or the inhaled spores of environmental fungi such as *Aspergillus fumigatus*, to reach the alveolar surface (24,25). Thus, an additional level of control is necessary that can protect from the invasion of pathogens via this route. Therefore humoral and cellular immune mechanisms are in place that directly act on the inner surface of the alveolus. Here a soluble (humoral) form of defense is mainly mediated by surfactant, while cellular immune mechanisms are more complex.

Surfactant is a compound mixture of ~90% phospholipids and 10% proteins. Its main function in terms of respiratory physiology is to lower the surface tension of alveoli by forming an extremely thin layer (Fig. 1D), which is on average only 200 nm thick, but on particular spots can be as thin as 90 nm (26,27). Without the reduction of surface tension the alveoli would collapse due to internal forces resulting from the tight curvature. This condition – respiratory distress syndrome (RDS) - is seen e.g. in preterm infants, where the transport machinery of surfactant is not yet functional. It can be treated by surfactant replacing therapies, the availability of which from the early 1990s on has led to a massive decline in RDS-related infant mortality (5).

An important physical effect of the thin surfactant layer is that it compresses cells lying under its surface to a very flat shape. This is especially pronounced for immune cells localized on the surface of airways and alveoli (1,26), as the alveolar flat shape strongly contrasts the more compact appearance of immunocytes in tissues. This fact is often overlooked, when lungs are prepared for microscopic analysis by tracheal instillation rather than perfusion fixation. Tracheal fixation leads to a loss of surfactant from the alveolar surface and thus an unnatural rounding of cells during the preparation. In contrast, perfusion fixation slowly fixes alveolar cells from the capillaries behind and thus nicely preserves the extremely flat morphology of alveolar macrophages (28). In the light of this fact, phagocytosis events of immune cells on the alveolar surface must be considered a 2-D event, which has important functional consequences, as we have shown and will be discussed in detail below (29).

In the conducting airways surface forces of surfactant transport particulate matter from the rigid surface (gel phase) of the surfactant layer into the underlying more liquid sol phase which is directly in contact with the mucociliary border of the epithelium. The presence of particles in the sol phase facilitates their mucociliary transport (30).

Interestingly, while the overwhelming majority of papers agrees on the model of surfactant as a thin film covering the entire alveolar surface there is also a competing model which claims that instead the surfactant forms closed bubbles inside alveoli (31). Although the argumentation and presented experimental data for this concept appear quite convincing, it has not been accepted by the field and the model is mainly discussed as an artifact of preparation. Clearly, bubbles made of surfactant would have interesting physical consequences on cellular processes going on in alveoli, thus a novel attempt to directly visualize surfactant *in vivo*, probably involving the now available intravital 2-photon imaging in breathing lungs (32), would help to settle this issue.

Next to its functions in the regulation of surface tension surfactant also contains four types of proteins (SP-A to SP-D), of which three have important immunological functions. Among them are the binding of bacterial LPS by or the direct absorption of surfactant proteins to the surface of pathogens. Surface binding of surfactant proteins can lead to pathogen aggregation and direct killing or the increase of the phagocytosis and killing activity of attaching immune cells. In addition, surfactant proteins can also interfere with dendritic cell (DCs) maturation or inhibit T cell proliferation, thus having an immunoregulatory function (reviewed in (5)). Furthermore, surfactant proteins have an immunomodulatory function, as their absence leads to the deviation of a protective Th1 into a non-protective Th2 response during pulmonary hypersensitivity reactions against *Aspergillus* antigens (33).

Immunological control of alveolar integrity – Immune cells

Next to the surfactant both, airways and alveoli as well as the interstitial tissue in between contain a multitude of cells from the innate and adaptive arm of the immune system. They will be briefly introduced here and their function will be discussed in detail below.

Bronchoalveolar lavage shows the presence of mainly alveolar macrophages (AM) in the air-exposed lung parts of non-infected mice, while shortly after infection with *Aspergillus fumigatus* there is a massive recruitment of neutrophils, which are also essential for controlling the infection (34). In lung tissue also many DCs can be found, that can sample the inner surface of the alveolus with membrane protrusions (28,32,35). While the extravasation of leukocytes in peripheral tissues has been extensively studied in the past and has led to the classical rolling-firm adhesion-transmigration model (36), emigration of leukocytes into alveoli seems to deviate from this model. In humans each alveolus is covered by up to 1,000 capillary segments. They form tight contact zones with the epithelium of the alveolar wall (Fig.1 B/C). The contacts have different levels of thickness, being as little as 200 nm at their thinnest points which is hardly more than the two cell membranes of the type I AECs and the endothelial cell with a sheet of shared basement membrane in between (37).

Importantly, the usual process of tethering and rolling of leukocytes that extravasate in peripheral tissues appears not to be necessary in the lung. Due to the large number and extremely thin diameter (2-5 μm) of capillary segments in alveoli neutrophils are naturally slowed down and even stopped in the lung blood vessels, which can occur independent of selectins. The actual transmigration event, although going over a very short distance of $\sim 1 \mu\text{m}$ is still very complex. Cells have to cross the endothelial layer, a basement membrane and

then the alveolar epithelial layer from the tissue side towards the air-exposed lumen of alveoli (reviewed in (37)). It has been postulated, that fibroblasts in the interstitial tissue provide a guidance cue to transmigrating leukocytes and that preferred exit sites are characterized by pre-formed holes in the basement membrane (37). The presence of preformed holes and guidance cues by interstitial pericytes has recently also been directly shown for extravasation of neutrophils into inflamed peripheral tissues (38,39).

Naturally, cells must strictly avoid to get in contact with the breathing air directly, which would otherwise immediately kill or at least completely inactivate them by desiccation (40). Thus we hypothesize, that leukocytes transmigrating into the alveolar lumen will squeeze themselves under the surfactant film from behind without ever getting in touch with the air filled lumen and flatten out during the process (Fig. 1D/E). Also DCs that have been shown to sample the alveolar lumen with cellular processes (32) should always remain below the surface of the surfactant film during this process. Given the fact that surfactant pulls particulate matter under its surface towards the hypophase (30) also make it appear unnecessary for a DC to sample the free air space, as it should be free of material. Movies made in lung explants did not show this process in a physiological manner, as here the lung slices were filled with liquid medium (24,41). Thus a novel approach using intravital microscopy of breathing lungs and focusing at the transmigration event of leukocytes with simultaneous visualization of the surfactant layer should be performed to clarify this issue.

While immune cells do enter alveoli via the intraseptal capillaries as described above, especially antigen presenting cells cannot easily leave the area again via lymphatic vessels. This is due to the fact, that under normal conditions there are only very few lymphatic vessels around alveoli themselves (28). This condition can change when the lung is set under chronic stress, e.g. hyperoxia. Then the extensive generation of lymphatic structures around small bronchioles, blood vessels and also partly around alveoli can be observed, but this condition is transient and vanishes when the stressor is taken away (42). They usually start at the regions where acini feed into larger bronchioles and also lymph nodes are only found along the branching points of the large airways and the trachea (28). Consequently it has been shown that DCs sample the alveolar lumen with membrane processes, but a functional interaction with T cells leading to activation happens in larger airways. Thus, DCs have to migrate through the intraseptal connective tissue to sites of lymphatic drainage that then leads them to the draining lymph nodes (32).

Pulmonary innate immune responses following fungal infection

The microbial mass inhaled by breathing air mainly consists of bacteria and some viruses but there is another important type of organism that constantly enters the airway system: microbial fungi. Based on the immune status of the host fungi can cause many different diseases of which some are more of an allergic type but also severe systemic infections are well known in clinics all over the world (43,44).

One of these human pathogenic fungi is *Aspergillus fumigatus*. This ubiquitous mold can be found in all climate zones on earth from desert areas (45) to the antarctic regions (46). In its asexual life cycle *A. fumigatus* produces a huge amount of 2-3 μm small spores (conidia) that are covered by a thin hydrophobic protein layer (47). These fungal particles are distribution morphotypes and are easily spread with any airflow. Unfortunately, due to their small size and hydrophobicity conidia can be taken up by all air breathing animals very efficiently. Although they have to pass the filter network of the airways the spores reach deepest areas of the lung tissue (Fig. 2). Despite the fact that the “natural role” of *A. fumigatus* as saprophyte is to degrade organic plant material this species harbors an assortment of pathogenicity factors that enables the fungus to invade and infect animal hosts under certain conditions. As described above the main entry port for this fungal species in air breathing animals is the lung. The following chapters will demonstrate which elements of the vertebrate immune system usually care for the clearance of the fungal invader and which factors predispose an organism for the development of an invasive aspergillosis.

Fungal recognition

If an immune cell is supposed to exhibit a certain response towards another entity it somehow has to recognize its presence. Regarding immune responses this recognition is realized by immune receptors. The vast majority of these membrane-bound or soluble molecules belong to the group of pattern recognition receptors (PRRs). By binding to a conserved pathogen-associated molecular pattern (PAMP) these receptors guide an activation signal into the cell and provoke a certain cellular response.

The protection against fungal infections like an invasive aspergillosis demands a well regulated orchestration of innate and adaptive immune responses. Fungal uptake (48), recruitment of effector cells by release of pro-inflammatory cytokines (49) and dampening immune overreactions by release of anti-inflammatory cytokines (50) need to be strictly governed. The basis for this regulation process are PRR-mediated cell reactions. Useful targets for such PRRs in fungal infections are compounds of the very species- and often

morphotype-specific cell walls which usually are composed of branched β -glucan, chitin and mannan networks (51,52). It is of note that immune receptor biology is very complex and at current state poorly understood. Derived from data of many works on different species it has become clear that the same receptor molecule might induce different cell reactions depending on the cell type on which it has been expressed (53-55). There are other hints that the cellular response of different immune cells might be dependent on the environmental conditions e.g. we have shown that neutrophils, macrophages and dendritic cells possess varying phagocytic capacities which are dependent on a) the spatial arrangement of the environment (2D versus 3D) and b) the kind of pathogen that they encounter (29). Interestingly we found a positive correlation between the ability to take up fungal particles and a spatial experimental condition that mimics the “natural” situation for a certain host-microbe interaction as closely as possible. These findings might be explained by the re-arrangement of immune receptors under the influence of environmental conditions but this concept needs further experimental validation.

Regarding the immune receptor situation in an *A. fumigatus* infection it has to be mentioned that the inhaled fungal conidia are hardly detectable by immune cells under resting conditions as their cell wall components are covered by a thin surface layer of the hydrophobic protein *RodA* (25). Nevertheless resting spores can be efficiently taken up by all classical phagocytes like neutrophils, macrophages and dendritic cells (29). For cells of the monocyte lineage (macrophages and dendritic cells) the type II C-type lectin DC-SIGN (Dendritic cell-specific ICAM-3-grabbing nonintegrin; CD209) was shown to bind and mediate *A. fumigatus* ingestion through the Raf-1 kinase pathway (56). Further, DC-SIGN seems not to directly initiate any inflammatory cytokine production but it is involved in modulating TLR-induced cytokine release (57). It is not present on neutrophils.

One explanation for the uptake of resting conidia by neutrophils might be the actions of members of the TLR family. Luther and co-workers were able to detect β -glucans on conidia by weak immunofluorescence signals although the spores were in their dormant state, implicating that they were fully covered by a masking *RodA* layer (53). Although Aimaniananda *et al.* reported that the protein layer very efficiently abrogates immune responses (25) the antibody staining visualized that specific binding to cell wall components through the *RodA* hull is possible. Several works have shown that TLR4 is highly connected to *A. fumigatus* immunity. TLR4 expression is up-regulated in polymorphonuclear neutrophils (PMNs) upon exposure to all resting or activated morphotypes and PMNs deficient in TLR4 displayed an impaired phagocytic activity and fungal killing (55). These findings might indicate a possible

role for TLR4 in the uptake of resting spores after contact to murine neutrophils. In contrast Wang *et al.* reported that rather *A. fumigatus* hyphae than conidial particles are sensed by TLR4 (and CD14) on macrophages (58) whereas another work by Luther *et al.* highlighted the importance of TLR2 for the fungal uptake by murine macrophages (53). Beside the receptors TLR 2 and 4 the TLR signaling-specific adaptor protein myeloid differentiation primary response gene (88) (MyD88) is an important factor for initiating immune reactions towards *A. fumigatus* (59). Upon immunosuppression with cyclophosphamide and intranasal infection TLR4 and MyD88 k.o. mice die sooner and the fungal load in lungs of TLR2 and 4 in MyD88 k.o. mice is much higher compared to wild type animals. It is commonly believed that the main cellular response to TLR triggering is the synthesis and release of pro-inflammatory cytokines (60). The mediation of these responses to protect an organism from immune overreactions is very complex and has been reviewed by Arancibia (59). Interestingly other studies suggest that TLRs induce an anti-inflammatory response. Netea *et al.* demonstrated that germination of fungal spores can be regarded as an immune escape mechanism. From their data they draw the conclusion that conidia induce an inflammatory cytokine pattern via recognition by TLR2 and 4. As soon as the spores start to form germ tubes fungal binding to TLR4 is interrupted and the new signaling situation exclusively by TLR2 triggers the cells to produce IL-10 (61). Another Toll-like receptor which is involved in *A. fumigatus* immunity is TLR9 (62,63). TLR9 is believed to sense the fungal DNA and it exhibits its functions on endosomal membranes (64). Although this intracellular localization implies a delayed response towards the pathogen Ramaprakash *et al.* described a set of important reactions of antifungal responses modulated by TLR9 (65). Derived from their work TLR9 is involved in neutrophil recruitment to the site of infection. TLR9^{-/-} mice survive a challenge with swollen conidia significantly longer than wild type animals which might be due to a decreased lung tissue injury after an impaired neutrophil recruitment. Nevertheless the mortality rate in TLR9^{-/-} mice is the same as in TLR9^{+/+} animals. Under neutropenic conditions TLR9 k.o mice are more susceptible to an *Aspergillus* infection. As an explanation they stated that TLR9 is required for full Dectin-1 expression. Dectin-1 is another C-type lectin-like receptor (66) which is known to be involved in mediating anti-*Aspergillus* immune reactions (see below). Although many studies show that Toll-like receptors have a significant role in fighting fungal infections there are other works that doubt their importance (54,67). However also in clinical mycosis patients a positive correlation between polymorphisms in TLR genes and the development of invasive aspergillosis was found (62). Thus, at present TLR biology in fungal infections is still a matter of ongoing research. Just recently the impact

of TLR1 and TLR6 in the setting of an *A. fumigatus* infection has been demonstrated, convincingly showing that the antimicrobial TLR network is acting cooperatively in modulating immune responses and that we are far away from understanding this complex system (68).

Dectin-1 as another PRR is also tightly connected to anti-*Aspergillus* immunity and it can be mainly found on the surface of professional phagocytes including macrophages, dendritic cells and neutrophils (69,70) but also on bronchial epithelial cells (71). Deficiency of this molecule in mice leads to an elevated susceptibility in a model of pulmonary aspergillosis (72). Also mutations in or deletion of the Card9 gene, a central adaptor molecule in the Dectin-1 signaling pathway, renders mice more susceptible to fungal infection (73,74). The target PAMP of Dectin-1 is β -glucan (75) and as shown for the TLRs before the initiated cellular response is dependent on the cell type. Triggered on DCs Dectin-1 is able to directly induce inflammatory cytokine and chemokine production whereas the binding of Dectin-1 expressed on macrophages to β -glucan structures alone is not able to initiate cytokine release (76,77). Besides driving cytokine and chemokine synthesis conidial recognition by Dectin-1 on murine macrophages can lead to ingestion of the fungal spores. This process is inhibitable by pre-treatment of the immune cells with laminarin as competitor to fungal β -glucan structures (53). The work of Gersuk *et al.* (78) describes that Dectin-1 is a potent key receptor for murine macrophage phagocytosis and activation but only upon binding to swollen conidia or germ tubes and not to resting conidia. They also stated that the uptake of zymosan can be completely blocked by a pre-treatment of macrophages with laminarin but production of macrophage reactive oxygen species (ROS) is just partially influenced by laminarin. This might indicate that receptors with different PAMP targets than β -glucan are involved in the synthesis of reactive oxygen intermediates. This publication also shows that Dectin-1 is cooperatively inducing cellular responses together with TLR2 and TLR4. Collectively the concept that PRRs after encountering their specific molecular pattern are inducing pre-shaped cellular responses is outdated. Many recent reports make clear that receptor mediated pathogen recognition should be regarded as an interplay of more than one detection molecule (72,79,80). A very interesting view on Dectin-1 was delivered by a work of Faro-Trindade *et al.* (81), recently. This group found that an inflammatory response to resting *A. fumigatus* is delayed compared to swollen morphotypes. They proposed that resting conidia first have to be taken up by phagocytes (e.g. macrophages) and have to undergo an intracellular swelling process. Innate signaling pathways are subsequently activated by Dectin-1 that is located inside membranes of phagolysosomes.

Finally, there is a heterogeneous group of soluble factors in *Aspergillus* immunity. It was shown that the complement system gets activated upon infection with fungal spores (82,83) and that opsonization with complement factors facilitates phagocytosis of conidia by macrophages and neutrophils (83,84). A second, very important protein that acts as opsonin is the long pentraxin 3 (PTX3). Moalli *et al.* found this molecule to mediate *Aspergillus fumigatus* recognition and internalization by murine neutrophils under *in vitro* and *in vivo* conditions (85). The binding of PTX3-opsonized particles to immune cells is established by interaction of PTX3 and FC γ receptors (FC γ R). This interaction in turn activates complement receptor 3 (CR3) thereby inducing the phagocytic uptake of complement component 3 (C3)-opsonized *A. fumigatus* conidia. PTX3 is of great interest as it is stored in secondary granules of neutrophils to ensure a rapid release in response to inflammatory cytokine signals and TLR engagement (86,87). For several microbes it was shown that surfactant proteins have an immunological opsonization effect that can enhance phagocytosis (88,89). In contrast a recent study found that murine BAL surfactant does not have an effect on the pulmonary inflammation and the phagocytic uptake of resting conidia by murine macrophages (81).

Alveolar Epithelial cells (AECs)

The first type of respiratory epithelial cells that is encountered by inhaled spores of *A. fumigatus* are the tracheal epithelial cells. The main role for this epithelium is to filter inhaled air and to transport particles that get trapped inside the surface lining mucus out of the airways. For this type of cells it is of course not important to establish solid cell-cell interactions with inhaled entities. This characteristic behavior was proven by Paris *et al.* in an *in vitro* system with *A. fumigatus* conidia that did not display stable contacts to a cell layer that was grown of primary rabbit tracheal epithelium (90). The epithelial layer of the alveoli as blind alley of the airways is constantly exposed to inhaled foreign particles. In the last decade many works have shown that AECs have far more functions than just building up the alveolar backbone. In the study by Paris and co-workers they further isolated AEC type II cells from rats and co-incubated them with *Aspergillus* spores. After 6 h they found up to 45 % of conidia in close contact with AECs and spore uptake was frequently observed. Several other reports describe a set of PRRs that can be expressed on the surface of alveolar epithelial cells among which the Toll-like receptors constitute the biggest group (91-93). That these receptors are functional and have the potential to initiate pro-inflammatory immune responses has been elucidated in several studies on pneumonia caused by viruses and bacteria (reviewed in (94)). In contrast Mayer *et al.* showed that AECs might also be involved in

dampening of immune reactions (95,96). Here bronchial epithelial cells released soluble factors that down-regulated TLR-mediated production of pro-inflammatory cytokines under steady state conditions. In addition they demonstrated that these cells limited the pro-inflammatory responses of innate immune cells within the lung by building up an IL-10 dominated microenvironment. Regarding the situation upon pulmonary *A. fumigatus* infection AECs are described to induce pro-inflammatory immune reactions mediated by interferon- β (97). It is of interest that in this work exclusively resting conidia, which constitute the vast majority of inhaled fungal spores, and not swollen morphotypes are recognized by AECs. Although most of the projects dealing with the modulation of immune responses by AECs found TLRs to be involved in pathogen recognition Balloy *et al.* unraveled the existence of an IL-8 driven inflammatory immune response that is not controlled by MyD88 (98) which indicates that this novel pathway is not induced by TLR engagement. Sun *et al.* found Dectin-1 to be expressed on bronchial epithelial cells and reported that its expression was TLR2 dependent. The presence of Dectin-1 was necessary for the production of pro-inflammatory cytokines, reactive oxygen species (ROS) and antimicrobial peptides (71).

Macrophages

As soon as inhaled spores of *A. fumigatus* reach the alveolar lumen they will be physically trapped inside the thin surfactant film. This is the place where they encounter immune cells for the first time. It is widely accepted that alveolar macrophages (AMs) are the first cell type getting in contact with inhaled particles. Between 1980 and 2000 many mycological works demonstrated that macrophages can take up and kill conidia very efficiently, thereby preventing the fungus from the production of hyphae, the dangerous, invasive morphotype of *A. fumigatus* (99-102). At first view this process is straight forward and almost self-explanatory. However, a closer look at macrophage biology in the lung reveals that this type of immune response is far more complex.

It is commonly believed that macrophages develop from macrophage/DC progenitors (MDPs), a subset of proliferating cells in the bone marrow (BM) (103). Still within the bone marrow MDPs differentiate to monocytes which display CD115 (receptor for macrophage colony stimulating factor (CSF-1R)), CD11b (Mac-1) and low levels of F4/80 (EGF-like module-containing mucin-like hormone receptor-like 1) on their surface. At this developmental state the myeloid phagocytes leave the BM and enter the blood stream to give rise two different major monocyte subpopulations.

The first one is designated as inflammatory monocyte. At least in adoptive transfer experiments it was shown that this cell type is more of a short-lived nature (104). Inflammatory monocytes are characterized by a CD115⁺ CD11b⁺ F4/80⁺ appearance and high surface expression of the chemokine receptor CC-chemokine receptor 2 (CCR2), the cell adhesion molecule L-selectin (CD62L) and the myeloid marker lymphocyte antigen 6C (Ly6C). The fractalkine CX₃C-chemokine receptor 1 (CX₃CR1) is rarely found on these cells (105). Due to this phenotype inflammatory CCR2⁺ CD62L⁺ LY6C⁺ CX₃CR1^{low} monocytes can be efficiently recruited to sites of infection by following a vascular CC-chemokine ligand 2 (CCL2) gradient (104). As source for CCL2 under lung infection conditions other monocyte subsets (106) or even epithelial (107) or endothelial (108) cells might serve. After entering the inflamed lung tissue this subset of monocytes can be regarded as so called classically activated macrophages (CAMs or M1 macrophages). Local CAM activation is induced by interferon gamma (IFN- γ) together with a microbial TLR trigger (109,110). M1 macrophages are of a strong pro-inflammatory nature secreting cytokines like IL-1, IL-6, IL-12 and IL-23 (111). This cytokine pattern clearly shows that the important role of M1 macrophages is to initiate T_H1 and T_H17 responses. In the course of these immune reactions different effector cells of the innate arm of the immune system like neutrophils are recruited and their antimicrobial action potential is promoted. Besides exhibiting phagocytosis and releasing further pro-inflammatory cytokines, these cells (112,113) together with the M1 macrophages (114) also produce and secrete highly reactive oxygen and nitrogen intermediates and radicals. The purpose of these reactive compounds obviously is to directly attack microbes and kill them but their mode of action is absolutely unspecific. A severe damage of the surrounding tissue is closely associated with the oxidative response. To counteract the destructive pro-inflammatory consequences the establishment of an anti-inflammatory milieu is needed. In recent studies it was shown that M1 macrophages can convert into such a regulatory phenotype (115,116). For this conversion usually two stimuli are required. The first trigger comes from immune complexes, prostaglandins, adenosine or apoptotic cells which are encountered by the macrophage. Together with a second stimulus that often is derived from a TLR ligand the macrophage is re-programmed to synthesize huge amounts of IL-10 and to down-regulate IL-12 production. This dramatic change of cytokine pattern promotes T_H2 responses which in turn favor the generation of alternatively activated macrophages (AAM, wound healing or M2 macrophages) by release of IL-4 and IL-13 (117,118). Macrophages that are treated *in vitro* with IL-4 and IL-13 show significantly reduced production of pro-inflammatory cytokines and oxidative compounds (119). Furthermore they

have the ability to control the proliferation of surrounding lymphocytes (120) and to secrete components of the extracellular matrix thereby helping to restore tissue homeostasis (121,122). Interestingly, AAMs produce several proteins with chitinase activity, indicating that these cells besides their regulatory function also have direct antimicrobial effects on fungi (123-125). The relevance of AAMs in an *Aspergillus fumigatus* infection has been demonstrated by Bhatia *et al.* recently (126). They found that pulmonary macrophages of an AAM type could already been observed 6 h after onset of an intratracheal infection.

The first type of macrophages that comes into contact with inhaled entities is a group of cells that is referred to as tissue macrophages. Inside the lung two major subsets of these resident cells can be distinguished, lung macrophages and alveolar macrophages. Both subsets are derived from the second major monocyte population in the peripheral circulation. These monocytes are well definable by the specific surface marker expression CCR2⁻ CD62L⁻ Ly6C⁻ CX₃CR1^{hi} F4/80⁺ CD11c⁻ CD11b⁺ (104,127,128). Intravital microscopy revealed that this cell subset is crawling in and around the vascular endothelium in an LFA-1 dependent manner and has the ability to enter and reside in uninfected tissue (128). Inside the lung parenchyma they undergo a maturation process to lung macrophages in which they up-regulate CD11c expression on their surface and simultaneously lose CD11b (127). This cell stage might be regarded as an intermediate phenotype. In 2007 Landsmann *et al.* were able to demonstrate that lung parenchymal macrophages are precursors of an even further developed phenotype, the alveolar macrophage (129). In the same work they showed that both tissue resident cell types are capable to proliferate *in situ* to replenish this macrophage pool over time. Until today it remains unclear if after tissue injury, infection and/or inflammation the resident cell pool is exclusively filled up by local proliferation or if also monocytes of the blood stream have an impact in this process.

Regarding the biological role of alveolar macrophages as resident tissue macrophages it is assumed that they generally have a regulatory character although our knowledge about these cells is very fragmentary at present. In a recent review article Murray and Wynn mentioned that “tissue macrophages [...] suppress inflammation mediated by inflammatory monocytes thereby ensuring that tissue homeostasis is restored following infection or injury” (130). Resident tissue macrophages occur at sites where heavy pro-inflammatory immune reactions might constantly be induced and they could be regarded as important cell type for the regulation of immune responses. For example the gut is a main entry port for pathogens that are taken up with the food. For colonic macrophages it was shown that they build up an IL-10 saturated milieu that protects the gut flora from inflammatory responses (131,132).

Macrophages that reside in the marginal zone of the spleen have the capacity to mediate self-reactivity to apoptotic cells (133) that constantly appear during cell degradation in this organ. Jenkins *et al.* found that pleural and peritoneal tissue macrophages responded to IL-4 treatment with an *in situ* proliferation and for peritoneal macrophages they described a resulting M2 phenotype (134). Such M2 macrophages in turn were found by Bhatia *et al.* to inhibit asthma symptoms associated with an *A. fumigatus* infection (126). Further there are hints that alveolar macrophages can also mediate adaptive immune reactions. Holt *et al.* showed that these cells are able to down-regulate the antigen presenting capacity of dendritic cells (135).

Taken together the facts about macrophages and their subsets it is getting clear that their biology is very complex. Although many subtypes with distinct functions have been described it is well known that macrophages possess the ability to switch from one functional phenotype to another depending on environmental signals (136,137), that macrophages share their progenitors with dendritic cells (DCs) and that even macrophages can give rise to cells with a DC-like phenotype under appropriate conditions (104,138,139). The regulatory mechanisms for this plasticity are still not well understood and are matter of many ongoing research projects.

Nevertheless it can be summarized that pulmonary macrophage populations are more than just phagocytic cells and that they have many more duties than to eliminate inhaled organic and inorganic particles by ingestion. Unpublished *in situ* 2-photon microscopy data of our group lead to the idea that phagocytosis might not be a major task for macrophages in the lung. Compared to other cells e.g. neutrophil granulocytes, they are localized at certain spots in the alveoli where they take up fungal spores but they are not very mobile. In infected lung tissue neutrophils patrol throughout the whole organ thereby taking up every conidium that is accessible. These observations might indicate that the main function of neutrophils in the alveolus is to clear the organ from microbes and that macrophages are regulatory cells for initiating and controlling the recruitment of the granulocytes. In fact it was also shown by Mircescu *et al.* that macrophages but not neutrophils are dispensable for fungal clearance (34).

Neutrophil Granulocytes

In contrast to other cell types of the myeloid lineage like macrophages and DCs the major part of neutrophil maturation takes place in the bone marrow. This is also the location where most of the cells reside subsequently. Under steady state conditions less than 2 % of the whole

neutrophil population is circulating in the bloodstream (140) where they have a half-life of 6 – 11 hours (141,142). Under conditions of infection or sterile inflammation pro-inflammatory cytokines and chemokines induce a process that is called neutrophil mobilization which can elevate the murine blood leukocyte number from less than 10 % to more than 30 % (143). Inside the bone marrow this process is described to be dependent on a granulocyte colony-stimulating factor (G-CSF) induced CXCL1 (KC) and CXCL2 (Mip-2) chemokine gradient that chemotactically guides the neutrophils from the bone marrow into the blood stream (143,144). Once they pass the site of inflammation inside the vascular system they sense another panel of chemoattractants (tumor necrosis factor (TNF), IL-1 β , chemokines, lipid mediators) which is secreted by sentinel cells such as resident macrophages inside the inflamed tissue (59,145). In order to pass the endothelial barrier neutrophils usually have to engage a series of physical interactions that are known as leukocyte adhesion cascade (146). As mentioned earlier the situation inside the lung might be different and neutrophil velocity gets already extremely slowed down by the very narrow capillary system. At the end of the stopping process the neutrophils transmigrate through the epithelium either by a transcellular or a paracellular route depending on the modulation of neutrophils and endothelial cells by certain chemokines (147). The last hurdle neutrophils have to take in their recruitment process is the perivascular basement membrane. This step and required mechanisms are not very well understood at current state. Some ideas are gathered in a review article by Sadik *et al.* (148). Inside the target tissue neutrophils are commonly guided to the site of inflammation by following CCR2/CXCR2 ligand gradients (148). In a murine infection model of pulmonary aspergillosis a loss of the pattern recognition receptor Dectin-1 resulted in significantly decreased levels of IL-1 α , IL-1 β , TNF- α , CCL3/MIP-1 α , CCL4/MIP-1 β , and CXCL1/KC which in turn were accompanied with an insufficient lung neutrophil recruitment and uncontrolled fungal growth (72). A second, very recent study on Dectin-1 k.o. mice revealed lower IL-23, IL-17A und IL-22 levels in lungs of infected animals (149). In subsequent experiments this group took a closer look at IL-22^{-/-} mice and also found decreased IL-1 α , TNF- α , CCL3/MIP-1 α and CCL4/MIP-1 β concentrations and impaired neutrophil recruitment compared to wild type animals.

Once a neutrophil has reached the site of infection it exhibits its repertoire of antimicrobial actions. This type of neutrophil is more active than neutrophils in the blood stream in terms of cytokine production (150). By secretion of chemoattractants they send danger signals throughout the body to get help from inflammatory cells, e.g. more neutrophils and macrophages (151-153). A second contribution of neutrophils to fungal clearance is

represented by direct interactions between the immune and the microbial cells. Neutrophils are able to take up fungal particles by TLR-mediated phagocytosis (154) and efficiently kill morphotypes that have lost the outer *RodA* layer (155,156). Besides the uptake of fungal morphotypes neutrophils are known to simply attach several conidia to their surface (29). In addition these spore-decorated PMNs tend to form huge cell aggregates in infected lung tissue (41,157). This observation together with the formation of neutrophil extracellular traps (NETs; see below) constitutes effective control. Pathogens get entrapped in a sticky mass of DNA with antimicrobial character and subsequently they are surrounded by neutrophils that prevent the microbes from spreading until the infectious mass is resolved. The hallmark of granulocytes (eosinophils, basophils and neutrophils) are their granules which make this group of immune cells unique in their mode of action towards pathogens. Granules are intracellular vesicles which are filled up during neutrophil maturation inside the bone marrow with a heterogenic pattern of antimicrobial and membrane-replenishing substances (158,159). They can be sub-divided by electron microscopy, various staining techniques, by cell fractionation and by their different functions into two major groups. Primary or azurophil granules mainly contain proteins or peptides which are directly involved in pathogen killing. Secondary or specific granules in turn help to replenish membrane components and mediate free radical reactions (160). Granulocytes are able to secrete their granular contents into the environment. This provides the cells with the capability to directly attack extracellular pathogens that are too huge to be phagocytosed (160). With elastase and cathepsin G two serine proteases present in azurophilic granules have been described which are tightly connected to *A. fumigatus* immunity (161). The major molecule found in specific granules is lactoferrin. Under co-incubation conditions with conidia and human primary neutrophils Zarembek *et al.* detected this substance in the cell culture supernatants (162). They further correlated the appearance of lactoferrin to a significantly reduced fungal growth in their system and hypothesized that this antimicrobial effect might have been developed due to a binding of accessible iron to lactoferrin. This could have deprived the fungus of this element which is essential for its growth. In 2007 Jaillon *et al.* reported that the soluble pattern recognition receptor PTX3 is also stored in specific granules providing a fast mechanism to modulate phagocytic events (86). In concert with degranulation activated PMNs undergo a process that is characterized by enormous oxygen consumption, called respiratory burst. The key enzyme of this reaction is NADPH oxidase a complex of multiple sub-units that generates superoxide radicals by transferring electrons from NADPH across membranes and coupling them to molecular oxygen. Upon activation the enzyme complex is established around the

flavocytochrome b₅₅₈ which, under resting conditions is mainly found inside the membranes of specific granules (163). However during respiratory burst the NADPH oxidase is exclusively found around phagosomes or attached to the cytoplasmic membrane around a neutrophil (164). A second important enzyme involved in oxidative responses by neutrophils is myeloperoxidase (MPO) which is highly present in azurophilic granules (160). It generates hypochlorous acid (HOCl) and chloride anions (Cl⁻) from hydrogen peroxide (H₂O₂). Both enzymes exhibit their direct antimicrobial potential mainly inside the phagolysosome during intracellular degradation of ingested pathogens (165). Nevertheless the products of their enzymatic reaction, reactive oxygen species (ROS) are also released into the environment where they develop their direct toxic potential to microbes (165). It is of course very likely that this mode of action not only effects microorganisms but also host tissue. The destruction of organ tissue by overreacting neutrophils is a very central point (166,167) which should always be taken into account when the virulence of a certain pathogen is assessed. Besides their more or less unspecific anti-microbial function ROS are also known as signal mediators in inflammatory responses. The current state of knowledge was reviewed by Lee *et al.*, recently (168). To speculate on the relevance of the oxidative burst for the immunological fight against *Aspergillus fumigatus* it was shown for both key enzymes of that process, NADPH oxidase and myeloperoxidase, that they are significantly involved in survival of infected mice (169,170). A very effective neutrophil response towards invading pathogens has been described by the group of Zychlinsky in 2004 (171). They unraveled a novel cell death mechanism called NETosis in which neutrophils upon stimulation with different bacterial and fungal (24) microbes are able to explosively release their nuclear and/or mitochondrial (172) DNA into the environment (Fig. 3). The histone decorated nucleic acid as such appeared to be very sticky to microorganisms what provided this structure with the ability to entrap microbial cells. Based on this observation Zychlinsky and co-workers designated this filamentous mesh as neutrophil extracellular traps (NETs). They further found that NETs are decorated with a variety of antimicrobial compounds like myeloperoxidase, neutrophil elastase, lactoferrin and PTX3 (86,171). As all these factors have been described to mediate *Aspergillus* immunity the relevance of NET formation in respect to fungal infections was of great interest. In 2010 we addressed this question by a murine *in situ* 2-photon microscopy approach (24) and we found that upon intratracheal infection with swollen *Aspergillus* conidia NET formation by neutrophils was present inside all lung lobes. Interestingly we were not able to observe a direct antimicrobial effect of NETs towards resting or swollen fungal morphotypes indicating that for clearance of tissues from this type of pathogen subsequent immune responses might

be crucial. Meanwhile the process of NET formation has been shown to be also relevant in other lung inflammatory diseases. Papayannopoulos *et al.* found NETs inside the sputum of cystic fibrosis patients and they described their production to be relying on an initial chromatin decondensation by neutrophil elastase (173,174). A very recent publication claimed that NETs have the capability to directly induce epithelial and endothelial cell death mediated by histones and myeloperoxidase (175). This process might contribute to the severe cellular destruction in organs with a strong neutrophil infiltrate.

In the vertebrate host defense against *A. fumigatus* neutrophils are regarded as key cell population. Their relevance is underlined by the clinical facts that neutropenic patients (176) and people that suffer from defective neutrophil specific enzymes (chronic granulomatous disease, CGD) (177) are of high risk to establish an invasive hardly treatable form of aspergillosis.

Adaptive immune responses to viral infection in the lung

As shown above the defense against pathogenic microorganisms by the innate immune system in the lung is very effective. However, the triggered responses are not always sufficient to completely prevent an inhaled pathogen from replicating and establishing an infection in the respiratory tract. Therefore, successful protection in many cases additionally depends on an adaptive immune response which is characterized through specific recognition of antigens derived from the respective pathogen. This recognition can on the one hand be conferred by antibodies which are produced by antigen-specific B cells and bind the pathogen, leading to neutralization and clearance. On the other hand, specific T lymphocytes are able to recognize pathogen-derived antigens displayed by infected host cells via the MHC I complex. These lymphocytes are mainly cytotoxic T cells that trigger the specific elimination of infected host cells, thereby preventing further replication, spread and persistence of intracellular pathogens. Equally important, the adaptive immune system provides a memory function protecting the organism from recurring infections with the same pathogen. A prerequisite for establishing an adaptive immune response is antigen-uptake by antigen-presenting cells (APC) at the site of pathogen encounter, i.e. the airways and lung. Subsequently APC migrate to lymphoid organs, i.e. the nasal or bronchus associated lymphoid tissue (NALT, BALT respectively) to present the antigens to lymphocytes. Those carrying the appropriate specific receptor will finally be activated and proliferate. Next to the classic professional APCs such as DCs a possible role for airway and alveolar epithelial cells in antigen-presentation and the induction and maintenance of adaptive responses is increasingly recognized. Also our understanding as to

where in the respiratory tract antigen-uptake and -presentation as well as priming and maintenance of specific lymphocytes take place is constantly increasing.

In primary pathogen encounter, especially the clearance of intracellular pathogens such as respiratory viruses depends on an effective adaptive immune response. Therefore the description and discussion of our current knowledge regarding the mechanisms of adaptive immune responses in the respiratory tract will largely be based on observations made during studies of primary respiratory viral infections, especially with influenza A virus.

Target cells of respiratory viral infection

Having passed the mechanical and chemical barriers of the respiratory tract mucosa, viral pathogens depend on specific receptors expressed on the host cell surface. These receptors enable viral binding and entry as a prerequisite for infection and vary depending on the pathogen. In the case of influenza A virus, which binds to sialic acid on the host cell surface, the primary target cells for replication are epithelial cells of the mouth and nasopharynx. Following first rounds of replication, the released viral particles are able to spread further along the respiratory tract infecting cells of the airway and eventually alveolar epithelium (178-180). The pathology of respiratory viral infection not only depends on the viral pathogen, its target cells and its mode of replication. Also varying strains of one pathogen, such as influenza A virus, show substantially altered characteristics in host pathology (180,181). Traditionally, the spreading of viral pathogens along the airways has been characterized through histological examinations of lung specimens from animals sacrificed at defined time-points following experimental infection or from human biopsies and autopsies (179-181). Lately, systems for *in vivo* imaging in experimental animals and the creation of viruses carrying reporter genes have opened new possibilities for monitoring pathogen spread and host response processes (182,183). Analysis of mice infected with a recombinant influenza A virus carrying GFP has demonstrated that indeed the infection successively spreads from the large conducting airways to lower parts of the respiratory tract (183).

Even though epithelial cells are the first targets for influenza A virus replication, alveolar macrophages and dendritic cells also possess receptors that allow their direct infection (184-186). However, it is unclear whether influenza A virus replication in macrophages and DCs contributes to viral propagation through release of new viral particles. It is of note that the capacity of influenza A viruses to infect macrophages is dependent on the viral strain and that this has clear consequences on the course of the disease (184,185,187). As alveolar macrophages can also transport antigens to the lymphoid organs and act as antigen-presenting

cells, also the induction and shape of the adaptive immune response is possibly affected (188). Therefore, we should keep these strain dependent differences in mind when evaluating animal studies using only single model pathogens or a single strain of a certain pathogen. Like influenza viruses, measles virus is taken up through the respiratory tract and replicates primarily in airway epithelial cells. However, unlike most influenza strains, measles virus can cause systemic infection and infected leukocytes are discussed as a mechanism of the virus to cross epithelial and endothelial barriers within the host (189).

As viral pathogens quickly replicate within host cells, the timely induction of an adaptive immune response to specifically identify and eliminate infected cells is crucial for the resolution of respiratory viral infections.

Antigen-presentation and induction of T cell responses in the lung and draining lymphatic tissue

Lung DCs are the main players in antigen-uptake in the respiratory tract as well as antigen-presentation in the lymphoid organs, thereby representing the interface between innate and adaptive immunity. Due to their localization below the respiratory epithelium and their dendritic extensions which protrude into the airway lumen, they come into contact with the external environment and are able to take up antigens and carry them to the internal lung microenvironment (reviewed in (190,191)). Since the mechanical and chemical barrier functions of the respiratory tract epithelium prevent most antigens from reaching the lower airways, large numbers of DCs are associated only with the conducting airways while smaller numbers of DCs are present around the alveoli (192).

In mice, there are different subsets of lung DCs with phenotypical as well as functional characteristics. These include the CD103⁺ and CD11b^{hi}CD103⁻ resident mature DCs, which differ in their localization in the lung tissue and their capability to produce pro-inflammatory mediators and chemokines during pulmonary inflammation (193-196). CD103⁺ DCs localize at the mucosal surface in close contact with the respiratory epithelium, whereas the CD11b^{hi}CD103⁻ DCs are found in the lung interstitium. In addition to the mature (MHCII^{hi}CD11c^{hi}) lung DCs, monocyte-derived, phenotypically immature (MHCII^{low}CD11c^{low}) DCs are found abundantly in the lung interstitium. These have been shown to be poor activators of naïve T cells in viral infection but likely are the precursors for mature DCs (193,197). Another subset of dendritic cells found in the lung is that of plasmacytoid DCs (pDC), which were found to be the main producers of type I interferons during respiratory viral infections. Like the MHCII^{low}CD11c^{low} DCs, pDCs can migrate and

transport antigens to the draining lymphatic tissue but are only weak activators of naïve T cells due to limited expression of co-stimulatory ligands (198-200).

Due to practical issues, DCs subsets and their distribution and function in the human lung are less well characterized than in mice. Human DCs can be divided into migratory conventional DCs (cDC) and lymph-node resident DCs and further subsets are formed according to characteristic surface expression patterns of MHCII, CD11c and additional markers (reviewed in (190)).

Once lung DCs are triggered through the recognition of pathogen-associated danger signals and have at the same time taken up antigen from the external environment, they are mobilized and migrate to the draining regional lymphoid tissues via the afferent lymphatics (201)). This process is mediated by chemokines such as CC-chemokine ligand 2 (CCL2), CCL5, CCL20 and CCL21 released from respiratory epithelial cells as well as vascular and lymphatic endothelial cells (202-206). Production of these chemokines by surrounding cells is complemented by the up-regulation of various chemotactic receptors such as CCR7 and sphingosine-1-phosphate receptor on mature resident DCs in the inflamed lung, a process required for the egress to the draining lymphoid tissues (193,194,207,208). Activated respiratory DCs also up-regulate co-stimulatory and adhesion molecules as well as antigen-presenting molecules in order to exert their function as potent APC for naïve specific T cells upon contact with these. In mice, emigrant CD103⁺ lung DCs have been shown to transfer antigen to lymph node resident CD8 α ⁺ DCs and both populations are able to prime naïve T-cells. This transfer of antigen to a second DCs population enhances the number of antigen-presenting and T cell-activating DCs in the lymphoid tissue (209-211). Furthermore, in influenza A virus infection, CD8 α ⁺ DCs have been shown to migrate away from the draining lymph node to accumulate in the lung interstitium where together with other DCs subsets they are essential for the induction of an effective anti-viral CD8⁺ T cell response (212). Recent work demonstrates a role for such DCs - T cell interactions within the lung tissue also in an asthma model. T cells were found to accumulate in close proximities of the airways, where they were activated by antigen-carrying DCs (32).

The interaction between APCs, that have captured antigen in the lung, and naïve T cells, which are primed to confer specific adaptive immunity at the site of infection, occurs in the draining lymphoid tissues. These are either the draining lymph nodes or the bronchus-associated lymphoid tissue (BALT), which is a tertiary lymphoid organ induced in response to microbial exposure or other forms of lung inflammation (213). Antigen-carrying DCs reach the draining lymph nodes via the afferent lymph, whereas in addition to the lymphatic route

also direct migration across the epithelium has been implied for DCs carrying antigen to the regional BALT (214). Next to antigen-presentation to naïve T-cells in the T cell zones of BALT, DCs have been shown to play a crucial role in maintaining BALT structure in influenza A virus infection. In this study, elimination of DCs following clearance of the virus led to disintegration of BALT with adverse effects on protective humoral immunity to the virus (215). Up to now, the relative contribution of secondary lymphoid organs such as the lung draining lymph nodes and tertiary lymphoid organs such as BALT is not fully clarified. Many studies regarding BALT function were performed in mice lacking lymph nodes, leading to a potential overestimation of the contribution of BALT to adaptive immune responses. However, studies using alternative models or animals with lymph nodes could show that BALT and tertiary lymphoid organs in general can be immune inductive also in the presence of a functional immune system (reviewed in (216)).

Next to dendritic cells, which are the main antigen-presenting cells, also additional cell types present in the lung are able to take up and present antigens. These are either epithelial cells of the airways and alveolar space which present antigen locally or alveolar macrophages which, like DCs, are able to transport antigens and migrate to the draining lymph nodes. Even though antigen-transport and -presentation is well documented (188,217,218), it is still unclear whether and to which extent alveolar and also tissue-resident macrophages actually contribute to the induction of adaptive immune responses in the lymph nodes or lungs directly. Also, so far it has not been clarified whether direct infection of DCs and macrophages with influenza A virus affects their function as APCs and T cell activators (186).

Regarding epithelial cells of the airways and alveoli, it has been shown that AECII present antigen to CD4⁺ T cells via MHCII and promote the induction of regulatory T cells in the context of autoimmunity in mice *in vivo* (218). Also in models of mycobacterial and influenza A virus infection, primary murine AECII were found to up-regulate MHCII and to present microbial antigens (217,219). However, the contribution of antigen presentation by AECII to the induction of adaptive immune responses has so far not been clarified in detail. Nevertheless, the presence of such mechanisms implicates a role for the alveolar epithelium in adaptive immune responses beyond the release of cytokines and chemokines, also through direct interaction with T lymphocytes. This is especially interesting in the context of the recent finding, that influenza A virus infection induces cytolytic CD4⁺ effector cells that home to the lung (219).

Once antigen-specific T lymphocytes encounter APC in the draining lymphoid tissues they are activated, proliferate and differentiate into effector cells. In order to fulfill their function in the specific adaptive immune response, these cells leave the lymphatic tissues and migrate to the site of infection, i.e. the respiratory tract, via the bloodstream. Extravasation from the circulation into peribronchial tissue is a stepwise process of recognition of the chemokines expressed in the lung, subsequent loose adhesion and rolling on the endothelium, activation of integrins on the lymphocyte, firm adhesion to the endothelium and egress into the tissue, which is followed by further migration along the gradient of chemokines produced by other cells such as epithelial cells, macrophages and neutrophils present at the site of infection (206,220-225). Especially in the alveoli, this process is likely to be supported by the microarchitecture of the tissue with its dense distribution of narrow capillaries as described above (37).

Having migrated to the lung, also different adhesion molecules expressed by effector T cells dictate movement in the tissue, e.g. integrin $\alpha 1\beta 1$ or integrin $\alpha 2\beta 1$ (226). Originally, proliferation of effector lymphocytes to numbers capable of clearing the pathogen at the actual site of infection was thought to take place in the draining lymphoid tissues. However, several studies published throughout the last years have provided evidence that activated effector T cells proliferate in the respiratory tract and that they interact with IL-15 *trans*-presenting DCs in the lungs, which promotes their survival. This proliferation and sustained survival of effector T cells at the site of infection is further supported by co-stimulation through the CD27 - CD70 axis, strongly depending on the antigen dose present (227-230).

In order to specifically eliminate host cells which display pathogen-derived antigens, effector lymphocytes rely on a range of different mechanisms. Upon recognition, they exocytose perforin- and granzyme-containing granules, leading to the direct lysis of target cells, or they induce target-cell apoptosis through FAS ligand or TRAIL expression. Next to these cytolytic functions, virus-specific CD8⁺ T lymphocytes can produce large amounts of pro-inflammatory mediators such as interferon- γ , TNF and MIP1- α in response to the recognition of virus-infected host cells (reviewed in (231)). In the influenza model, it has been demonstrated that the target cell type presenting the antigen in the lung dictates the effector function performed by CD8⁺ T cells. Lung infiltrating CD45⁺ inflammatory mononuclear cells such as CD11c⁺ DCs trigger both cytotoxicity and the release of inflammatory mediators. This was shown to be modulated by the co-stimulatory ligands CD80 and CD86 expressed by the CD45⁺ inflammatory cells. In contrast, virus-infected CD45⁻ epithelial cells of the respiratory tract solely triggered CD8⁺ T cell mediated cytotoxicity without leading to

the release of inflammatory mediators. This was mirrored by the isolation of much larger numbers of interferon- γ producing CD8⁺ T cells from the lung interstitium than from the airspace (232).

Nevertheless substantial numbers of CD8⁺ T cells can be isolated from the airspace of influenza A virus infected mice by bronchoalveolar lavage (232). These cells must have left the lung parenchyma and, like e.g. alveolar macrophages and recruited neutrophils, reside within the airspace in only loose contact with the epithelium, protected from the environment solely through the surfactant. It is not clear however, whether the majority of these lymphocytes isolated from the airspace of influenza A virus infected animals have actively transmigrated through the epithelial lining or whether they are exposed as a consequence of epithelial destruction either through viral replication or cytotoxic immune effector mechanisms. As described above, neutrophil migration into alveoli occurs through directed crawling of the leucocyte along interstitial fibroblasts (37,233). Lymphocytes homing to the lung during respiratory infections employ a similar strategy for extravasation from the bloodstream, leaving them in the interstitium and in close basal contact with the epithelial lining (224,225). In this context it is interesting to note that analyses of differentiated, ciliated human airway epithelial cells showed that MHCI expression is polarized and restricted to the basolateral membrane (234). Assuming that this finding is also true for the murine respiratory epithelium, effector lymphocytes would not necessarily need to cross the epithelial barrier in order to recognize and eliminate infected epithelial cells which present antigens via MHCI. Nevertheless, antigen-specific T effector lymphocytes need to be present to some extent in the airways for the recognition of alveolar macrophages, DCs and also neutrophils which have been infected by respiratory viruses. Analyses of supernatant from a human airway epithelial cell line grown in a double chamber and incubated with *Staphylococcus aureus* showed that apical supernatant triggered enhanced chemokine receptor expression on both CD4⁺ and CD8⁺ T cells as well as enhanced chemotaxis of CD4⁺ T cells when compared to basal supernatant (235). These results clearly implicate a polarization of chemokine release by airway epithelial cells and a role for triggering transmigration of effector lymphocytes through the respiratory epithelium in inflammatory conditions. Another study could show that RANTES secretion by cultured human airway epithelial cells treated with interferon- γ preferentially occurred at the apical cell surface, again implicating the presence of epithelial cell-intrinsic mechanisms for routing T lymphocytes to the mucosa (236). In the context of chronic obstructive pulmonary disease (COPD), it was found that also a polarized trans-epithelial gradient of CXCL11 promotes lymphocyte egress into the airway lumen through the

intact epithelial barrier (237). Here it is interesting to note that we find CXCL11 to be up-regulated in transcriptional analyses of type II alveolar epithelial cells isolated from influenza A virus infected mice (unpublished results). The same study hypothesized that apically released chemokines can either slowly diffuse across the intact epithelial barrier or are transcytosed by the epithelial cells. Adhesion of T cells to the basal surface of the epithelial cells and migration across the monolayer caused small, localized increases in the permeability of the epithelium, facilitating paracellular movement of lymphocytes (237). An *in vivo* model in experimental influenza infection in mice has demonstrated a prominent role also for IL-15 in regulating trafficking of CD8⁺ T effector cells to the airspace (238). Most of the above mentioned studies however, have addressed their questions regarding respiratory epithelial polarization and its functional consequences for T cell trafficking in *ex vivo* cell culture models. Therefore future investigations using modern high resolution imaging techniques will be needed to further clarify some of these issues as well as their significance *in vivo*. Nevertheless, destruction of the epithelial barrier is a hallmark of influenza A virus pathology in animals and humans and is likely to enhance the presence of lymphocytes in the airspace beyond directed lymphocyte trafficking through the intact epithelial barrier.

CD8⁺ T lymphocytes are the major cytotoxic effector T cell population in charge of recognizing and eliminating infected host cells. In addition, research of the last years has delivered growing evidence for the possibility of CD4⁺ T cells to develop into effector cells with functions beyond lymphocyte help. They were found to migrate to sites of infection and to mediate strong immune protection independently of CD8⁺ T cells and B cells, not only through the production of cytokines but also through direct cytolytic activity (reviewed in (239)). This has been described for infections with various viral pathogens, recently also for respiratory influenza A virus. Here, infection induced cytolytic CD4⁺ effector lymphocytes that resided in the lung but not the draining lymph nodes, expressed granzyme B and lysed peptide-pulsed target cells in a perforin-dependent manner. When transferred, CD4⁺ T cells from the lungs of infected animals were able to protect from lethal influenza A virus challenge (219). This work is also one of the studies demonstrating evidence for the induction of MHCII on epithelial cells in the airways upon viral challenge, thereby providing targets for the cytolytic CD4⁺ lymphocytes generated in the course of respiratory influenza A virus infection. In line with this, recently a role of cytotoxic CD4⁺ T cells was also found in influenza A virus associated immunopathology (240).

In general, roles for cell-cell interactions in the respiratory tract other than those classically defined for maintaining homeostasis or coordinating immune responses are increasingly

recognized and here our knowledge is constantly growing. Thereby, we are also becoming more and more aware of the role of the special microenvironment of the lung. For example, resident alveolar macrophages adhere to integrin-expressing alveolar epithelial cells and are thereby in indirect contact with DCs in the alveolar wall. In addition, they may directly contact those DCs extending dendrites into the airway lumen from the interstitium. This close association allows cross-regulation between DCs's, macrophages and airway as well as alveolar epithelial cells and has implications not only during the described phases of the induction and exertion of respiratory immune responses but also during their regulation as well as the subsequent repair processes (241,242).

Regulation of effector lymphocyte function in the lung and regeneration following infection

Under healthy conditions, immune responses in the respiratory tract need to be tightly controlled in order to prevent excessive responses to harmless antigens. During infections of the lung, this control is loosened in order to enable the potent local responses which ensure pathogen containment and clearance. Next to their beneficial effects however, immune responses mounted during infections of the respiratory tract pose a threat to the health of both the organ and the organism. This threat through immunopathology arises on top of the damage *per se* caused by pathogen replication. Especially cytotoxic responses that target host tissue during viral infections can cause serious collateral tissue damage if not regulated correctly. Therefore, the immune system possesses several ways to tightly control anti-viral responses in the respiratory tract and to thereby protect the host from immunopathology. Nevertheless, the superior goal is the elimination of the infecting pathogenic microorganism, resulting in a balancing act between stimulating and suppressing specific immune reactions in the lung (reviewed in (243,244)).

Originally, regulatory T cells (T_{regs}) were thought to have roles in maintaining immune homeostasis mainly in the naïve host. However, T_{reg} cells can be induced during chronic inflammation and during infection, including viral infections of the respiratory tract (245,246). Lately, the T_{reg} response following influenza A virus infection was characterized in a mouse model and showed potent induction of these regulatory cells at the site of inflammation. Influenza A virus infection-induced T_{reg} cells were found to be highly suppressive towards $CD4^{+}$ and $CD8^{+}$ T cells *ex vivo* and it was suggested that they are able to inhibit effector responses in an antigen-dependent manner (247). Also data from respiratory RSV infection suggest a role for T_{reg} cells in preventing immunopathology in the lung, as their

depletion led to excessive inflammation and tissue damage (248,249). In contrast however, depletion of T_{regs} did not alter the course of disease following influenza A virus infection (250). Therefore further studies will be essential in order to improve our understanding on the exact contribution of regulatory T cells to the prevention of immunopathology in respiratory viral infection, how this protection is triggered and by which mechanisms it acts.

One way in which T_{regs} suppress immune responses, is by secreting substantial amounts of the anti-inflammatory cytokine Il-10. Next to T_{reg} cells, also CD4⁺ and CD8⁺ T cells present in the lung in response to influenza A virus, RSV or simian virus 5 infection produce large amounts of Il-10, suggesting that this is a general mechanism of preventing excess pathology in respiratory viral infection (251-253). The fact that this Il-10 production occurs mainly at the site of infection and not in the draining lymphoid tissues might be a hint for a role of the lung microenvironment in regulating this special feature of T effector cell function. Another suppressive cytokine constitutively expressed in the lung is TGFβ1. It plays a role for immune regulation in homeostatic conditions but is equally important in respiratory viral infections, where treatment with TGFβ1 suppresses immunopathology (but also impairs viral clearance (254)). In line with this finding, neutralization of TGFβ1 during influenza A virus infection strongly aggravated the inflammatory tissue injury (255).

Next to secreting TGFβ1, the respiratory epithelium regulates immune responses in the lung through the expression of CD200. This inhibitory molecule acts on alveolar macrophages, which express the negative regulator CD200 receptor (CD200R). A lack of CD200 was shown to result in enhanced sensitivity to influenza A virus infection, to lead to a delayed resolution of inflammation and to enhance pathological T cell responses (256,257). Next to epithelial cells, also apoptotic immune cells were shown to express CD200. This expression was up-regulated during the resolution phase of influenza A virus infection, therefore possibly contributing to immune inhibition once the virus has been cleared and the tissue needs to be protected from excessive damage (258).

Also innate immune cells contribute to regulation of anti-viral immune responses in the lung following induction of potent adaptive responses. In RSV infection, pDCs have been reported to inhibit immunopathology through an unknown mechanism (259) and in influenza A virus infection, neutrophils were found to protect from lung injury under certain conditions (260). Innate lymphoid cells, so far known to mainly contribute to immunity and inflammation in the intestine, were only recently shown to also accumulate in the lung following influenza A virus infection. Their importance in regulating lung homeostasis following viral infection was

demonstrated by depletion experiments, where airway epithelial integrity was lost and airway remodeling was impaired (261).

Taken together, many cellular and soluble factors are involved in regulating immune responses in respiratory viral infections and more research will be necessary in order to fully understand how they are orchestrated to allow optimal protection from both pathogenic cytopathy and immunopathology. Only a complete understanding will allow effective interventions in cases where there is dysregulation, without at the same time favoring pathogen replication and persistence.

A cleared respiratory viral infection can leave behind a substantially affected lung. The respiratory epithelium needs to proliferate in order to regenerate and fulfill its function as a barrier towards the environment. Signs of epithelial repair can be found in lung specimens from influenza infected human patients (179) and in experimentally infected animals (262). Only recently has it been clarified that lung regeneration following influenza A virus infection involves stem cells of the bronchiolar epithelium which undergo rapid proliferation and re-localize to areas of alveolar ablation to reconstitute the alveolar-capillary network (263).

While protecting the lung from excessive inflammation, an altered immune environment as well as structural environment with implications for the fight against challenges with secondary pathogens is created.

Consequences for secondary bacterial infection

Bacterial co-infections have for long been recognized as a frequent serious complication in patients infected with measles, influenza A virus and other respiratory viral pathogens (reviewed in (264)). The high death toll of the 1918/1919 influenza pandemic, predominantly caused by bacterial superinfections, dramatically highlights the impact of the viral/bacterial synergism on influenza morbidity and mortality (265). The fact that a substantial number of fatalities in the recent swine flu outbreak of 2009 could be attributed to bacterial co-infection demonstrates that what was recognized for the Spanish flu is not only a historic threat to human health (266).

The mechanisms underlying the synergism between influenza A virus and bacterial pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus* have been of strong interest for researchers ever since this phenomenon has been recognized. First explanations focused on the destruction of the respiratory epithelium through the viral infection, leading to exposure of the basal membrane and cryptic receptors facilitating bacterial adhesion. This is underlined by

the finding that following experimental influenza A virus infection in mice, bacteria preferentially adhere to areas of the respiratory epithelium damaged by the viral infection (264). Altered lung function during the viral infection further supports the bacteria in reaching these areas in the first place (264). Also the degree to which proliferation and repair of the respiratory epithelium following influenza infection occurs strongly contributes to the outcome of a secondary bacterial challenge, as demonstrated in a mouse model for pandemic H1N1(2009) infections (267).

Throughout the last decade however, a number of studies described a variety of additional mechanisms all suggesting a central role for immune modulation through viral infections in mediating aberrant anti-bacterial defense in secondary infection. This virus-induced immune-suppression at least in part explains the often observed long-lasting enhanced susceptibility to bacterial infections following influenza A virus infection. A sustained impairment of innate immune functions essential for the fight against bacterial infections was demonstrated to result in both increased bacterial transmission between hosts, facilitated colonization as well bacterial outgrowth in the respiratory tract.

It has been described earlier that respiratory viral infections lead to sustained alterations in the microenvironment of the respiratory tract. Expression levels of PRRs and scavenger receptors on lung resident macrophages are altered with distinct consequences for their functionality. Also the epithelium, when undergoing repair, expresses and exposes receptors different from those found in a healthy lung. DCs recruited to the respiratory tract in high numbers during the infection can be found there for an extended period of time even after resolution of the viral infection. This in turn has effects on antigen-presentation in secondary infections, in this case especially viral infections. In addition, there are long-term changes in the composition of the extracellular matrix as well as an increased distribution of blood and lymph vessels (reviewed in (268)).

Generally, many of mechanisms described for mediating suppression of anti-bacterial responses following respiratory viral infections are linked to those processes involved in regulating anti-viral responses and protecting from immunopathology in the primary viral infection. So, protection from adverse effects of the defense against a viral pathogen seems to take its toll when it comes to defending the host in concurrent bacterial infections.

As described above, the respiratory epithelium expresses CD200 in order to regulate the function of alveolar macrophages via CD200R (256). In secondary bacterial infection following influenza however, CD200 expression was found to have detrimental effects, as

mice deficient of CD200R showed limited bacterial load in the respiratory tract and decreased systemic dissemination of the bacteria in a co-infection model. The study concludes that the threshold for the activation of lung innate immunity is raised through enhanced CD200 expression by the epithelium and by apoptotic immune cells following influenza A virus infection and that this handicaps effective anti-bacterial defense in secondary infections (258). Respiratory viral infection also has strong direct effects on alveolar macrophages, which are the first line of defense against bacteria which have successfully entered the respiratory tract. AMs are desensitized to stimulation by different Toll-like receptor ligands, resulting in decreased cytokine production and NF-kappaB activation. This state persists for several months after experimental influenza or RSV infection in mice, which might be one explanation for the long-lasting enhanced susceptibility towards secondary bacterial infection as it can be observed also in human influenza patients (269). One documented mechanism by which influenza infection inhibits alveolar macrophage function is the down-regulation of scavenger receptor MARCO on the macrophages, mediated by IFN-gamma released during the viral infection and resulting in a loss of phagocytic function (270). Impaired NK cell TNF-alpha release in response to secondary bacterial challenge was found to be another upstream mechanism of inhibited alveolar macrophage function (271).

If alveolar macrophages are not able to clear a respiratory bacterial challenge, large numbers of neutrophils are recruited to the lungs. In secondary bacterial infection, alveolar macrophages were found to not be able to induce this recruitment of neutrophils, which further favors bacterial outgrowth (269). However, this is not a stand-alone mechanism for the development of severe secondary bacterial disease following influenza, as we could show that in naïve mice, neutrophils are dispensable for the defense against a low-dose pneumococcal infection which was lethal in influenza pre-infected mice (21). Nevertheless there is clear evidence that also neutrophils next to neutrophil independent mechanisms do contribute to bacterial superinfection following influenza (272). The question whether and how exactly aberrant neutrophil function plays a role in the synergism between respiratory viral and bacterial pathogens is just one example of how complex the situation is and how much the conclusions drawn from model infections can depend on the viral and bacterial strains used, their doses and the timing between the infections.

Mechanisms to control adaptive immunity in respiratory viral infection include the induction of T_{reg} cells as well as the production of anti-inflammatory Il-10 by T_{reg} as well as CD4⁺ and CD8⁺ effector T cells. There are contradictory reports whether the high levels of Il-10 present in virus-infected lungs affect anti-bacterial defense in secondary infection. On the one hand Il-

Il-10 was shown to be increased in co-infected mice as early as 6 days and also later, on day 14, following influenza infection (272,273) and treatment with anti-Il-10 antibody resulted in reduced bacterial outgrowth and mortality (273). On the other hand, only a mild importance for Il-10 was reported when analyzing Il-10 deficient mice in pneumococcal infection following influenza (274).

Hallmark cytokines produced during respiratory viral infections are type-I interferons. They are essential for anti-viral defense but also have adverse systemic effects on the host organism, such as profound lymphopenia (275). Even though the role of adaptive lymphocytes in defending the host against primary infection with respiratory bacterial pathogens such as *Streptococcus pneumoniae* has not fully been clarified (276), lymphopenia was discussed as a possible factor in enhancing susceptibility to bacterial infection following influenza (264,277). Even though a contribution of type-I interferons to severe post-influenza bacterial disease could clearly be demonstrated (274) we found forced induction of lymphopenia not to affect anti-bacterial defense in a model of pneumococcal infection (21). The possible role of the presence of virally induced T_{reg} cells in the lung of influenza infected hosts for anti-bacterial defense has not been addressed in detail before. However, in depletion experiments, we could not find an effect of T_{reg} cells on the outcome of secondary pneumococcal challenge following influenza (unpublished results).

Taken together, even though our understanding of how the processes induced during respiratory viral infection affect anti-bacterial defense in secondary infection is rapidly growing, there are contradictory reports regarding some of the proposed mechanisms and we are also largely unaware of the underlying triggering events. It will be interesting to find out, whether there are single defined super-ordinate triggering events leading to the downstream effects described here. These will be interesting targets for intervention and the largest challenge here will be to keep the balance between the potent elimination of the underlying viral infection, prevention of immunopathology through effector cells of the anti-viral response and efficient recognition and clearance of secondary bacterial pathogens.

Summary and future directions

Our knowledge regarding the induction, exertion and regulation of immune responses in the respiratory tract has grown extensively in the last decades. Many of these recent findings have brought up new questions. Research has described a whole number of leucocyte and lymphocyte subsets acting in immunity of the respiratory tract, with phenotypes and functions

of these cell types still growing. One main future challenge will be the systematic unraveling of how all these cells develop, how their functions are orchestrated and how they interact in protecting us from the harmful environmental threats we are constantly facing upon breathing. Fascinatingly enough, our respiratory immune system has evolved to ensure protection from all kinds of pathogenic harms including extracellular as well as intracellular bacteria, molds and viruses – while at the same time tightly regulating reactions to harmless antigens and host-tissue directed immune responses.

As a thought-provoking impulse we have summarized what we believe to be central questions posed by the research reviewed in this article.

- Structure of the surfactant layer in vivo
- Trafficking and function of immune cells in the surfactant lined alveolus
- TLR/PRR networking under distinct immunological conditions
- Molecular basis of viral-bacterial synergism in pulmonary infection
- Developmental and functional aspects of pulmonary macrophage/DCs subsets
- Impact of the distinct microenvironment in airways and alveoli on immune functions
- Immunological impact of epithelial polarization
- Role of epithelial cells as immune effector cells (e.g. as phagocytes)
- Role of epithelial cells in the induction and regulation of immune responses

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Legends to Figures:

Fig. 1 The principal setup of the respiratory tract and a hypothetical sequence of events during immune cell entry into alveoli. (A) The respiratory tree with its bronchi reaching terminal bronchioli and finally several acini. The cartilagenous exterior skeleton of the bronchiolar tree is shown in blue. One acinus is boxed and shown enlarged in (B). (B) A large number of alveoli (beige) constitute an acinus. The individual alveoli are stabilized from the outside by protein fibres (dark lines) and each show extensive vascular supply from oxygen poor (blue) to oxygen rich (red) blood. Only some blood vessels are shown. A terminal bronchiolus is shown (thick red tube with dark lines) leading into alveoli, which are cut open to see the interior. Please note, that also some alveoli are already attached to the bronchiolus outside of the acinal area. A part of the open alveoli is boxed and shown enlarged in (C). (C) is a view into a set of 9 alveoli cut open at ~ midline. Envision each opened alveolus in this view to be a concave surface with edges made by membrane contacts between type I AEC (pink). Type II AEC (beige) are scattered between the type I AEC and much smaller in surface. They produce surfactant, which covers the entire surface of the alveoli as a thin film. Please note the presence of numerous pores of Kohn in the paper plane (filled arrowheads) or leading towards the bottom of the paper plane into alveoli below the current surface (empty arrowheads). Pores of Kohn connect the individual alveoli, contain a continuous surfactant layer and can also be crossed by immune cells. For clarity, alveolar macrophages are not shown, but would be present at the alveolar surface. The alveolar septae (light brown) contain blood vessels (red), fibroblasts (light blue) and protein fibres (brown lines). One septum with a blood vessel is boxed and shown enlarged in (D). (D) shows the different cell and protein/surfactant layers between a neutrophil granulocyte in the blood vessel and the air volume of an alveolus. (E) shows from left to right a hypothetical sequence of events that lead to the emigration of a blood vessel neutrophil into the alveolus towards a particle (empty arrowhead), that is already submersed under the surfactant layer (green). Further details are discussed in the main text. Please note that the surfactant layer is bulging as the neutrophil enters from behind (filled arrowheads) but always remains closed to shield the neutrophil from direct contact with the breathing air. As a result, during and after the transit the neutrophil is pressed flat against the alveolar surface while engulfing the foreign particle. Experiments are needed to test, whether this scenario is really happening *in vivo*.

Fig. 2 Conidial uptake by type I alveolar epithelial cells (AECs). REM photographs of *Aspergillus fumigatus* infected murine lungs 6 h after onset of the infection. Conidia are presented in false color green. Please note the progress of spore ingestion following the pictures from up to down. In the lower picture conidia can be seen inside the cytoplasm of an AEC. Arrowheads point to shapes of erythrocytes in alveolar capillaries. Scale bars: 5 μm ; 5 μm and 10 μm , respectively.

Fig. 3 NET formation by human neutrophils after contact to *Aspergillus fumigatus* conidia. REM photograph taken 3 h after co-incubation of freshly isolated human neutrophils and resting spores of *A. fumigatus*. Scale bar: 2 μm .